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TECHNICAL REPORT

Cross-species virus-host protein-protein interactions inhibiting innate immunity

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July 2016

HDTRA1-13-1-0017

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Prepared by:

Hauptman-Woodward Medical Research Institute

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14. ABSTRACT
The single-stranded negative sense RNA (ssRNA(-)) virus families Arenaviridae, Orthomyxoviridae, and Paramyxoviridae include some of the most deadly emerging zoonotic pathogens. These viruses are known to impair antiviral interferon activation responses in humans through virus-host protein-protein interactions (PPIs) that disrupt intracellular signaling pathways. However, little is known about the role of homologous PPIs in animal (donor) hosts and the extent of adaptation necessary to form similar complexes in new (recipient) hosts. The present work seeks to characterize the importance of PPIs in species jumiping events.

15. SUBJECT TERMS
viral pathogen, zoonotic, arenavirus, host tropism, protein-protein interactions, RIG-I, Z protein, CARD domain, MAVS

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UNIT CONVERSION TABLE

U.S. customary units to and from international units of measurement*

U.S. Customary Units	Multiply by → / ← Divide by†		International Units
Length/Area/Volume			
inch (in)	2.54	$\times 10^{-2}$	meter (m)
foot (ft)	3.048	$\times 10^{-1}$	meter (m)
yard (yd)	9.144	$\times 10^{-1}$	meter (m)
mile (mi, international)	1.609 344	$\times 10^{3}$	meter (m)
mile (nmi, nautical, U.S.)	1.852	$\times 10^{3}$	meter (m)
barn (b)	1	$\times 10^{-28}$	square meter (m^2)
gallon (gal, U.S. liquid)	3.785 412	$\times 10^{-3}$	cubic meter (m^3)
cubic foot (ft^3)	2.831 685	$\times 10^{-2}$	cubic meter (m^3)
Mass/Density			
pound (lb)	4.535 924	$\times 10^{-1}$	kilogram (kg)
unified atomic mass unit (amu)	1.660 539	$\times 10^{-27}$	kilogram (kg)
pound-mass per cubic foot (lb ft^{-3})	1.601 846	$\times 10^{1}$	kilogram per cubic meter (kg m^{-3})
pound-force (lbf avoirdupois)	4.448 222		newton (N)
Energy/Work/Power			
electron volt (eV)	1.602 177	$\times 10^{-19}$	joule (J)
erg	1	$\times 10^{-7}$	joule (J)
kiloton (kt) (TNT equivalent)	4.184	$\times 10^{12}$	joule (J)
British thermal unit (Btu) (thermochemical)	1.054 350	$\times 10^{3}$	joule (J)
foot-pound-force (ft lbf)	1.355 818		joule (J)
calorie (cal) (thermochemical)	4.184		joule (J)
Pressure			
atmosphere (atm)	1.013 250	$\times 10^{5}$	pascal (Pa)
pound force per square inch (psi)	6.984 757	$\times 10^{3}$	pascal (Pa)
Temperature			
degree Fahrenheit (°F)	$[T(^{o}F) - 32]/1.8$		degree Celsius (°C)
degree Fahrenheit (°F)	$[T(^{o}F) + 459.67]/1.8$		kelvin (K)
Radiation			
curie (Ci) [activity of radionuclides]	3.7	$\times 10^{10}$	per second (s^{-1}) [becquerel (Bq)]
roentgen (R) [air exposure]	2.579 760	$\times 10^{-4}$	coulomb per kilogram (C kg^{-1})
rad [absorbed dose]	1	$\times 10^{-2}$	joule per kilogram (J kg^{-1}) [gray (Gy)]
rem [equivalent and effective dose]	1	$\times 10^{-2}$	joule per kilogram (J kg^{-1}) [sievert (Sv)]

*Specific details regarding the implementation of SI units may be viewed at http://www.bipm.org/en/si/.
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Grant/Award #: HDTRA1-13-1-0017
PI Name: L. Wayne Schultz (departed project Feb. 2015) and Timothy C. Umland
Organization/Institution: Hauptman-Woodward Medical Research Institute
Project Title: Cross-species virus-host protein-protein interactions inhibiting innate immunity

What are the major goals of the project?

List the major goals of the project as stated in the approved application or as approved by the agency. If the application lists milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion. Generally, the goals will not change from one reporting period to the next. However, if the awarding agency approved changes to the goals during the reporting period, list the revised goals and objectives. Also explain any significant changes in approach or methods from the agency approved application or plan.

Hypothesis. Viruses disrupting interferon induction in a donor host will disrupt this antiviral response in recipient host species using similar protein-protein interactions (PPIs) upon successful host range expansion.

Goal 1: To identify conserved cross-species virus-host PPIs by screening of proteins encoded by ssRNA(-) *Arenaviridae*, *Orthomyxoviridae* and *Paramyxoviridae* family members with histories of host jumps, including to humans, against interferon response pathway proteins RIG-I, MDA5 and MAVS from donor and known or potential recipient hosts (e.g., human, pig, chicken, horse, dog, cat, mouse, and bat).

Goal 2: To define and characterize virus-host PPI interfaces by combining structural models derived from small-angle x-ray scattering (SAXS) and homology models generated by bioinformatics analysis to help guide experimental mutagenesis studies that will verify key residues and conserved structural features necessary for protein complex formation.

Goal 3: To predict the presence or absence of important barriers to host species jumping in an expanded range of human and animal hosts by integrating bioinformatics and experimental data to cluster virus protein sequences capable of participating in similar PPIs that impair cellular antiviral responses.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results, including major findings, developments, or conclusions (both positive and negative); and 4) key outcomes or other achievements. Include a discussion of stated goals not met. As the project progresses, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Introduction

Emerging and re-emerging infectious diseases are a regular occurrence globally (Figure 1). The Zika virus is the latest example gaining widespread attention. Many of the (re-)emerging infectious diseases are viral in nature and involve a host species jump from animals to humans. Many factors may contribute to (re-)emergence of viral pathogens, including environmental changes, shifts in human population to new regions, and the presence of poor public health and reporting standards.

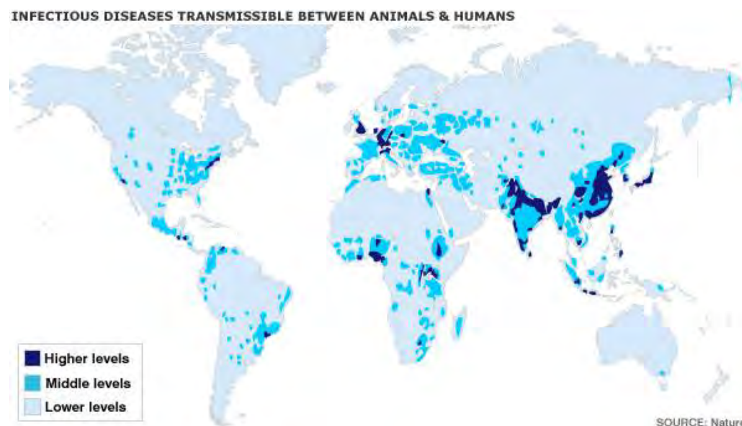

Figure 1. Zoonotic infectious diseases are a global problem.

This project focused upon members of three negative-sense single-stranded RNA (ssRNA(-)) virus families with know or suspected histories of changes in host-species tropism from animal to humans. In the *Paramyxoviridae* family, Hendra and Nipah (natural reservoir in bats), and Newcastle (natural reservoirs birds) viruses have histories of host species jumps, and are listed on HHS, USDA, or Overlap select agents lists. The *Arenaviridae* have natural reservoirs in various rodents but many are capable of infecting humans, with Guanarito, Junin, Machupo, Sabia, Lassa fever viruses listed as select agents. The *Orthomyxoviridae* influenza A has an extensively tracked history of host species jumps, including birds, pigs and other mammals, and humans.

Multiple factors typically are involved in determining if a virus successfully changes its host tropism, ranging from environmental factors to genetic changes. This project focused on the role antiviral interferon responses, and specifically the RIG-I mediated response, has in host tropism changes. Many viruses, including member of the *Paramyxoviridae*, *Arenaviridae*, and *Orthomyxoviridae* families, possess proteins that interact with the RIG-I pathway to modulate frontline antiviral responses, which can be important for establishing infection and/or modulating pathogenesis (Figures 2 and 3).

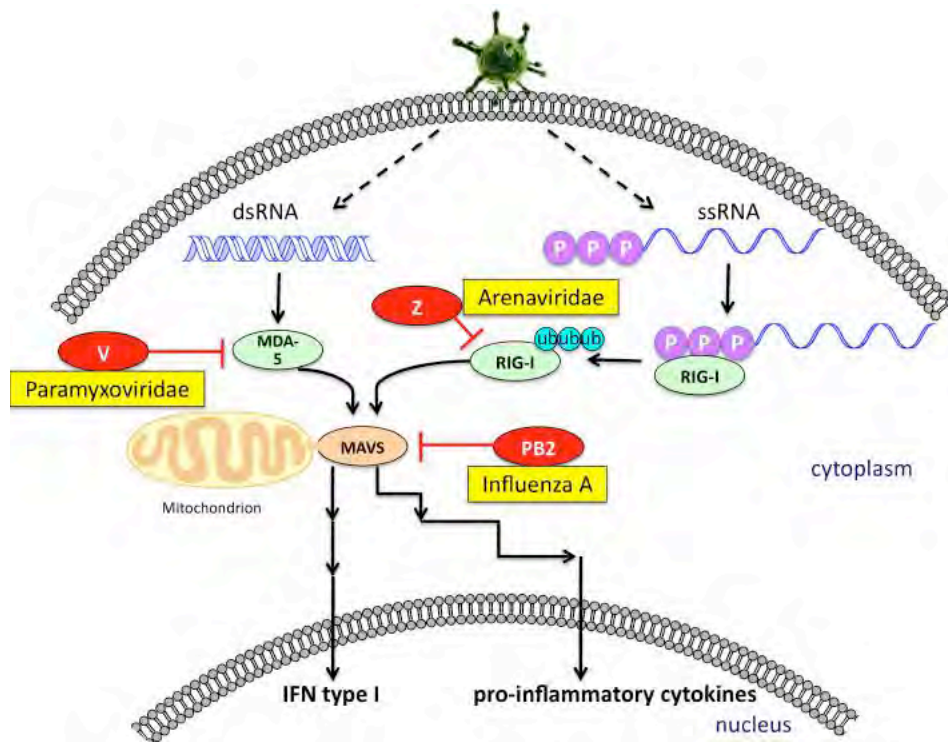

Figure 2. Schematic of several virus-host protein interactions within the RIG-I mediated interferon response pathway.

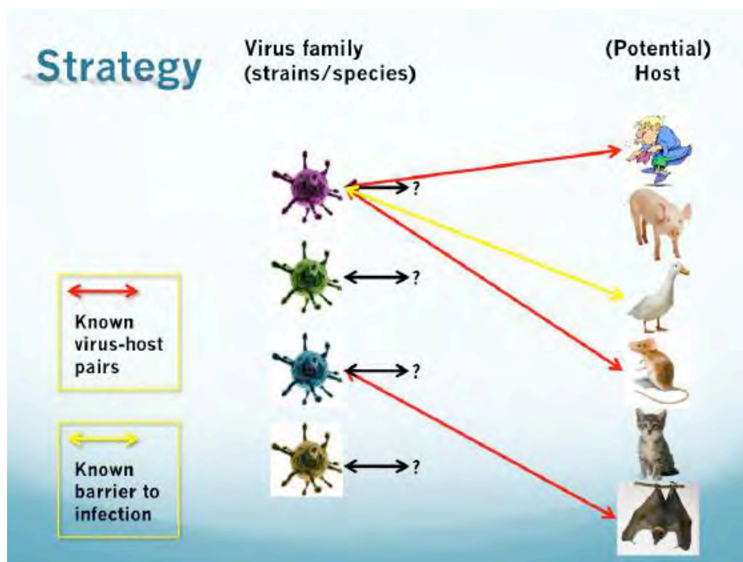

Figure 3. Schematic describing the overall project strategy of selecting specific virus protein – host protein interactions based on known interactions, to develop screens to determine if these protein-protein interactions serve as barriers to changes in host tropism.

Arenaviridae Z proteins disrupt interferon signaling by binding to RIG-I

Arenaviruses are enveloped ssRNA(-) viruses that can infect humans coming in contact with chronically infected rodents, the natural reservoir. Arenavirus infection may result in

hemorrhagic fevers with up to 30% mortality rates. Several are currently listed as Select Agents. However, arenavirus species exhibit distinct host profiles, with a number not typically pathogenic to humans.

RIG-I is a cellular protein that detects the presence short 5' triphosphate dsRNA as a marker of RNA virus infection. RIG-I mediated interferon β (INFB) induction is a major first-line innate immunity response against viral infection. The inhibition or avoidance of this initial innate immune response is a commonly occurring strategy evolved within many different viruses, although a number of different specific mechanisms are utilized. The Z protein produced by arenaviruses was reported to directly interact with and disrupt INFB induction by RIG-I (Lipkin et al., 2010. J Virol., 84:1785). Interestingly, in this report signaling was disrupted by Z proteins of the New World (NW) arenaviruses Guanarito virus, Junin virus, Machupo virus, and Sabia virus, while the Old World (OW) arenaviruses Lassa fever virus (LASV) and lymphocytic choriomeningitis virus (LCMV) Z proteins did not inhibit this signaling pathway. While a general interaction with RIG-I was clearly established, neither the interacting (sub-)domains (or residues) from the Z proteins or RIG-I were identified. Sequence analysis of the OW and NW Z proteins revealed an overall conservation for its core RING finger domain but an insertion near the N-terminus differs significantly between OW and NW arenaviruses, suggesting an explanation for the difference in affinity for RIG-I. An NMR structure of LASV Z protein shows a typical cross-braced RING finger motif with two Zn ions stabilizing loops involved in protein-protein interactions; however, the N and C-termini are disordered extended strands. In contrast, our covariance analysis mapped *hotspots* for protein interaction to the surface of the core ring-finger domain. Sequence conservation mapped onto 3-D structures also demonstrated that residues within the core are the most highly conserved, although this in part may be due to the necessity of retaining specific residues required for the coordination of the two zinc ions (Figure 4). Several positions within the disordered N- and C-termini are also highly conserved, and so their possible relevance should be considered as well. It is our goal to identify the surfaces of interaction between RIG-I and Z proteins and determine if differences between protein-protein interactions (PPIs) fully explain the difference in interferon activation between the OW and NW arenaviruses. These results will then be used to predict potential animal hosts subject to interferon suppression by Z proteins from different arenavirus species or strains.

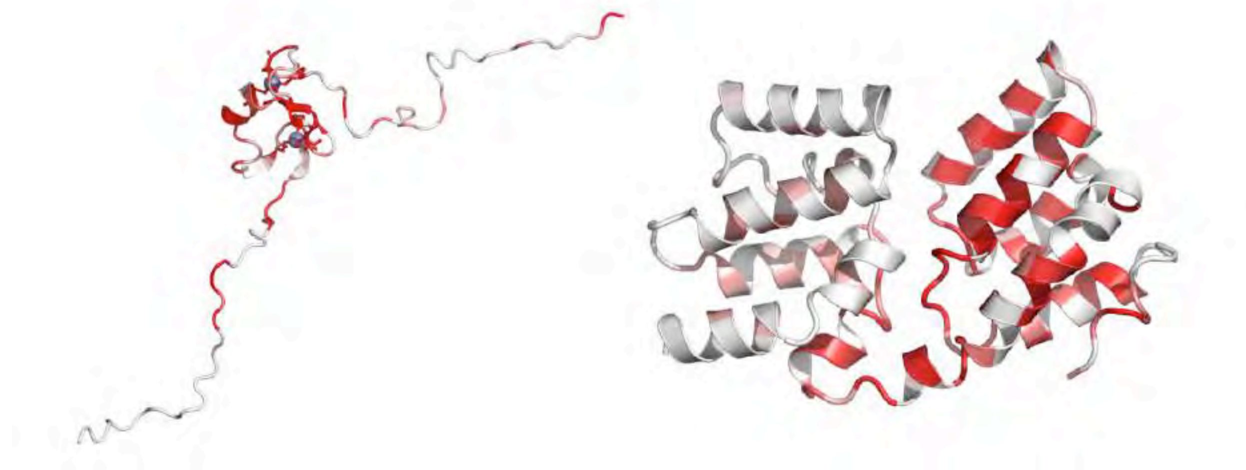

Figure 4. Sequence conservation mapped onto 3-D structures. LASV Z-protein (left) and the N-terminal double CARD of human RIG-I (right). Sequence conservation (red: highly conserved; pink: modestly conserved; white: poorly conserved) based upon multi-sequence alignments mapped onto these structures (sequences obtained from Swiss-Prot/TrEMBL). The high sequence conservation exhibited by the Z protein core RING finger domain may be due to the necessity of retaining residues to properly coordinate two Zn ions. The first (i.e., N-terminal) CARD of the RIG-I double CARD domain exhibits significantly more sequence diversity than the second CARD.

Arenavirus Z protein binding to RIG-I

We previously reported our development of a mammalian two-hybrid (M2H) assay to investigate the interactions of Z proteins from 4 species of arenavirus (Lassa, LCMV, Guanarito, and Junin) with differing constructs of human RIG-I. The natural reservoir for arenaviruses is mice and other small rodents, which have a RIG-I similar in sequence to human RIG-I. However, even a difference in one or two key residues may dictate the capability for a specific set of proteins to form a PPI. The M2H system works by detecting the interaction of two proteins via a split luciferase reporter. Multiple constructs encoding RIG-I (full-length, double CARD domain, helicase domain, and C-terminal RNA-binding domain; Figure 5) and arenavirus Z proteins for 4 representative species (OW: Lassa, LCMV; NW: Guanarito, and Junin) were individually subcloned into Checkmate M2H System (Promega) bait and prey reporter plasmids. The genes encoding the viral Z proteins were synthesized (Genscript), thereby avoiding isolating genetic material from potentially hazardous viral sources. These experiments suggested that with varying degrees of affinity, all of the arenavirus Z proteins bound to full-length RIG-I, with no significant distinction between the Z proteins from OW and NW arenaviruses. Testing for Z protein interactions with isolated domains of human RIG-I demonstrated all interacted with the double CARD domain, and Lassa and LCMV Z proteins were also capable of forming an interaction with the helicase domain. No interactions with the RIG-I C-terminal RBD were observed. The RIG-I double CARD domain is required to form a complex with the MAVS CARD domain to propagate the IFNB induction signal, and hence it not surprising that the Z protein

interacted with the RIG-I double CARD domain. However, these results did not agree with the differentiation observed (Lipkin, et al. 2010) between NW and OW Z proteins and RIG-I.

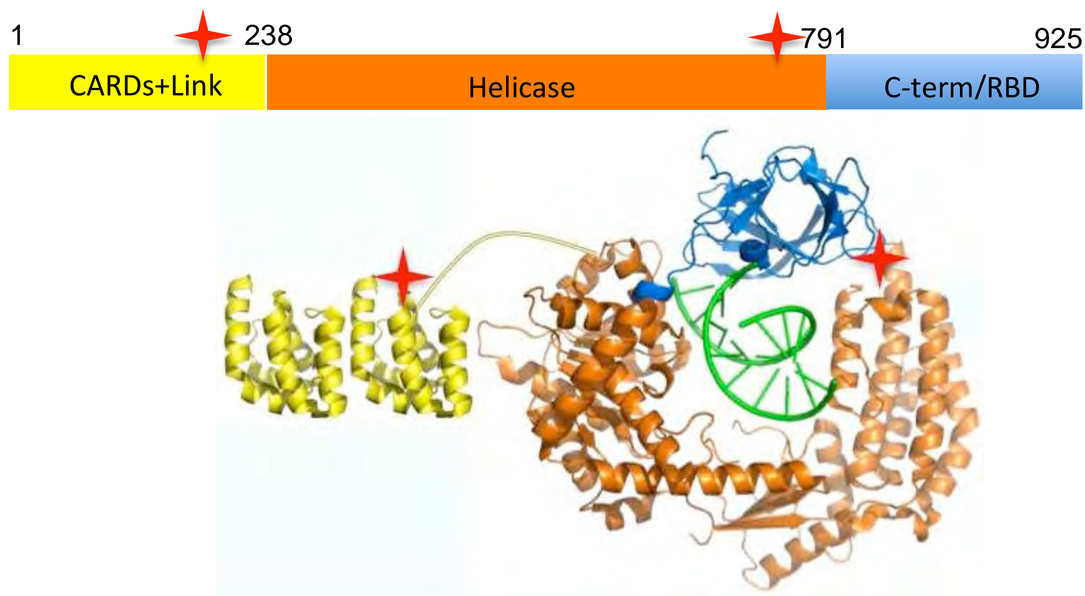

Figure 5. RIG-I domain structure. The N-terminus contains a double CARD domain and an intrinsically disordered linker sequence (yellow), followed by a helicase domain (orange), and finally, the C-terminal RNA binding domain (RBD) (blue). The red stars indicate important sites of ubiquitination by the E3 ubiquitin ligases TRIM25 and RNF135, with ubiquitination typically required for activation of RIG-I via inducing a conformational change that exposes the double CARD domain. The displayed 3-D structure is a model built from experimentally determined individual domains.

An improved PPI assay was developed over the past year. Bimolecular fluorescence complementation (BiFC) employs a split fluorescent protein (FP; a derivative of the Venus yellow fluorescent protein) optimized to provide a high signal-to-noise (s/n) ratio dependent upon the presence or absence of a specific PPI. The N-terminal and C-terminal halves of the FP are expressed as fusion constructs with the bait and prey proteins, respectively. Interaction of the bait and prey proteins results in complementation of the FP, producing a long-lasting, high s/n fluorescence that does not require the additional of exogenous reagents (Figure 6). This is unlike the luciferase-based M2H assay in which the signal is short-lived, requires addition of a luciferin reagent, and poorly detects potentially important but transitory PPIs.

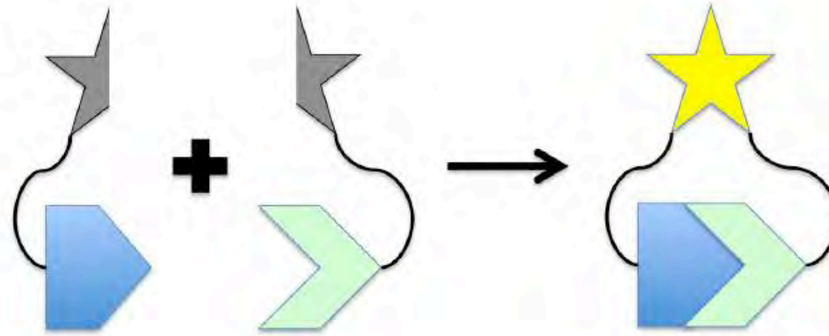

Figure 6. Schematic of the BiFC PPI assay system. Putative interacting proteins (e.g., prospective virus and host protein partners, colored blue and light green) are expressed in cultured mammalian cells in the context of fusion proteins (complementary N- and C-terminal halves of a fluorescent protein, colored grey). Formation of a PPI between the two test proteins results in formation of a functional fluorescent protein (yellow star), producing a high-level of fluorescence. The fluorescent protein has been optimized to minimize self-complementation in the absence of a PPI between the test proteins.

Appropriate BiFC plasmids were constructed to assay arenavirus Z protein interactions with human RIG-I constructs (full-length and double CARD domain; Figure 7). The BiFC assay indicated all four arenavirus Z proteins had a modest interaction with full-length human RIG-I, with no significant differentiation among the Z proteins. This observation was in agreement with the M2H assay results. It is known that RIG-I undergoes a structural change upon activation through sensing viral RNA, and this conformation change increases the exposure of the double CARD domain to solvent and hence to potential PPI partners. In both PPI detection assays, the full-length RIG-I is presumably primarily in the inactive state, with reduced accessibility to the double CARD domain. Thus, the observed modest Z protein – full-length RIG-I interaction matches expectations as the inactive full-length RIG-I conformation is likely not optimal for the formation of this PPI. Less expected is that all of the Z proteins (OW and NW representatives) exhibit very similar levels of interaction with the inactive full-length RIG-I. The BiFC assay detected varying degrees of Z protein – RIG-I double CARD domain interactions, with all producing a signal greater than that produced against the inactive full-length RIG-I. While all four Z proteins form a PPI with the double CARD domain, the two NW arenavirus representatives, Junin and Guanarito, display the extremes of the observed signal.

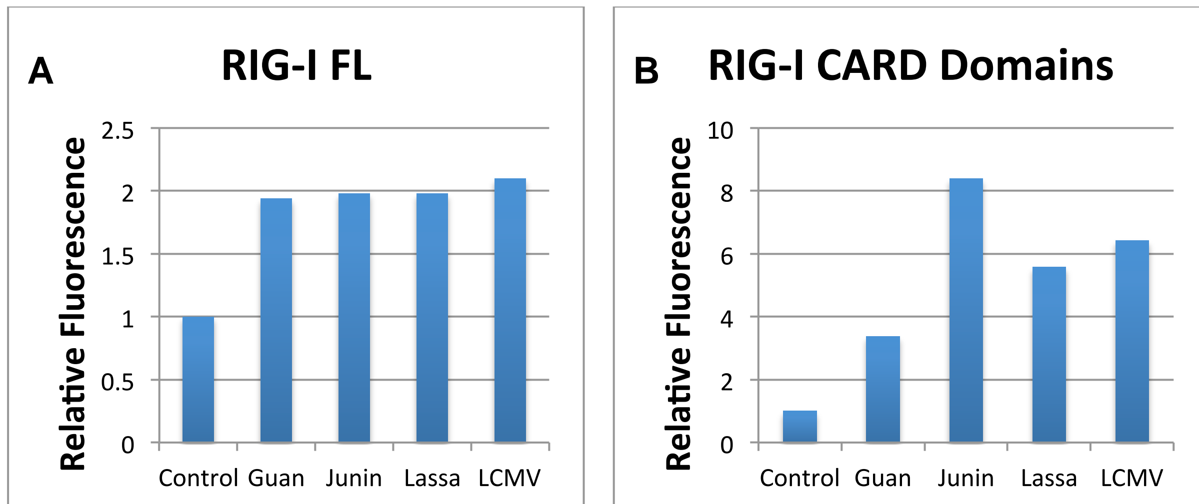

Figure 7. BiFC assay detecting interactions of arenavirus Z-proteins with human RIG-I as indicated by normalized fluorescence. Fluorescence activity occurs upon interaction of Z protein with RIG-I. Panel A: Full-length inactive RIG-I. Panel B: RIG-I N-terminal double CARD domain. The control is a measure of fluorescence in the absence of PPIs. Guanarito (abbreviated Guan) and Junin are NW arenaviruses and Lassa and LCMV are OW arenaviruses.

The report of Lipkin et al., 2010 suggested that OW arenaviruses do not down regulate INFB production through a Z protein – RIG-I PPI. However, a recent study found that arenaviruses classified as pathogenic to humans exhibited this Z protein – human RIG-I PPI, while those classified as non-pathogenic to humans did not form this PPI (Xing and Liang 2015. J Virol 89:2944). Our results are in agreement with this later report, as all of the Z proteins we tested are from human pathogens. We plan to test several Z proteins from representative non-human pathogens (e.g., Mobala virus (OW) and Bear Canyon virus (NW)) to confirm this trend, and to determine the molecular sites of interaction.

Virus sequence analysis. Sequences were collected from GenBank and Swiss-Prot/TrEMBL, and aligned using ClustalW. Phylogenetic trees for viral proteins were calculated with PhyML. While several residue positions are highly conserved across Z proteins (Figure 8), significant sequence variation is present (Table 1), but it is predicted that this occurs in the context of a conserved core three-dimensional fold (Figure 9). Of the highly conserved residues, many of those are required for either the direct coordination of the two Zn ions or to correctly structurally position these Zn coordinating residues. An N-terminal sequence variable insert is present in the NW arenaviruses, but otherwise there is a lack of an obvious sequence *fingerprint* to differentiate NW and OW arenavirus Z proteins. In fact, pairwise comparisons indicate that the Z proteins from with NW or OW arenavirus groupings are nearly as different in sequence versus comparison across NW/OW groupings. Several notable sequence differences are present in the Guanarito virus Z protein, and they may have a role in its decreased level of interaction with human RIG-I double CARD domain compared to other Z proteins tested.

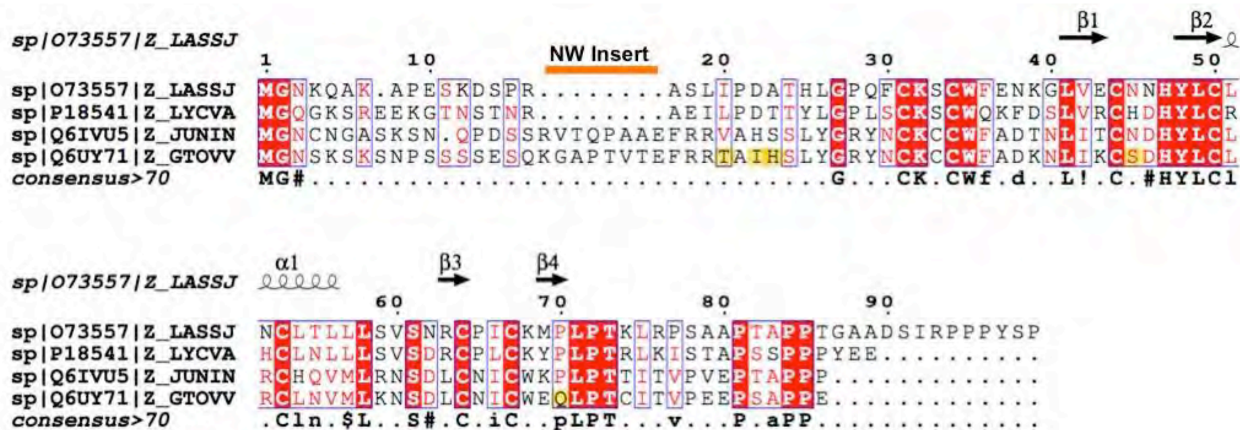

Figure 8. Multi-sequence alignment of arenavirus Z proteins thus far tested, with secondary structure annotation based on Lassa virus Z protein. Z protein code: LASSJ, Lassa virus; LYCVA: LCMV; Junin: Junin virus; GTOVV: Guanarito virus. Red shaded boxes indicate fully conserved residues, and unshaded boxes indicate similar but not fully conserved positions. The symbols in the *Consensus Sequence* (>70%) line are: uppercase is consensus level > 70%; lowercase is consensus level > 0.5; ! is I or V; $ is L or M; % is F or Y; # is N, D, Q, or E. Yellow shaded residues in the Guanarito Z protein sequence highlights notable variations which may account for this Z protein producing a lower fluorescence BiFC signal against human RIG-I double CARD domain compared to the other three Z proteins.

Table I. Conservation of Arenavirus Z protein sequences: % amino acid sequence identity*

	LCMV	Lassa	Mobala	BCNV	Guanarito	Junin
LCMV	100	52	49	40	38	36
Lassa		100	68	48	37	41
Mobala			100	46	37	37
BCNV				100	46	39
Guanarito					100	65
Junin						100

* Representative OW arenaviruses are listed on top, with representative NW arenaviruses listed below. Mobala virus and Bear Canyon virus (BCNV) are highlighted in yellow to emphasize that they have no history as human pathogens.

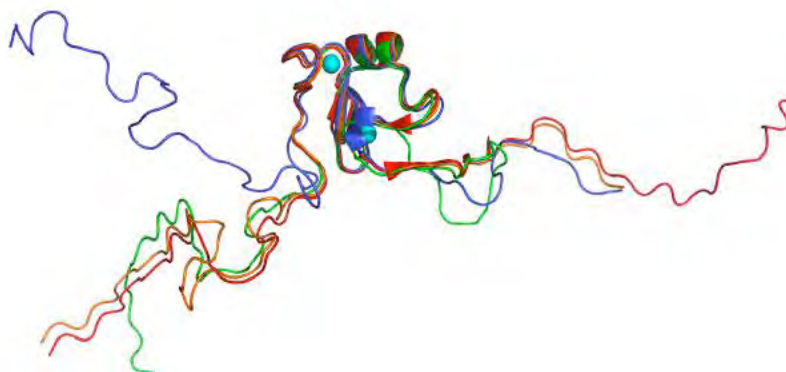

Figure 9. Superposition of LCMV (nmr) and Lassa, Guanarito, and Junin (models) Z proteins

Structural homology between Z proteins and host proteins TRIM25 and RNF135

Arenavirus Z proteins interact with the RIG-I N-terminal double CARD domain, as determined by both our M2H and BiFC assays. This same RIG-I domain participates with the single N-terminal CARD domain of MAVS, forming a PPI that propagates the IFNB induction signal. Thus, it is not surprising that a viral protein (e.g., Z protein) that interferes with the formation of a RIG-I – MAVS complex by sequestering the RIG-I partner in this interaction will have the effect of suppressing this innate immune response. Importantly, RIG-I must be activated prior to its interaction with MAVS. TRIM25 and RNF135 are E3 ubiquitin ligases that ubiquitinate RIG-I as part of this anti-viral signaling activation (Figure 10). Unexpectedly, despite low sequence similarity between Z protein and TRIM25 or RFN135, their core 3-D structures are predicted to be highly similar, with each possessing a RING finger domain coordinating two Zn ions. Presumably, TRIM25 and RNF135 each transiently interact with RIG-I during the ubiquitination process, and so it is plausible to propose that Z protein may mimic TRIM25's interaction with the RIG-I double CARD domain, and possibly RNF135's interaction with the RIG-I helicase domain.

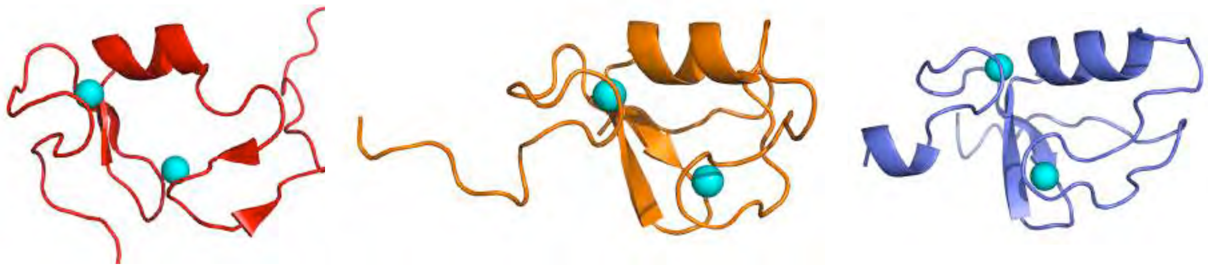

Figure 10. Comparison of Z-protein domain from Lassa (red), human TRIM25 (orange), and human RNF135 (blue). The Lassa structure was determined experimentally by NMR (PDB: 2M1S). The other structures are homology models based on similar structures. In all structures, both Zn binding sites are conserved.

Arenavirus Z protein interacts with MAVS CARD domain

Upon activation due to detection of viral dsRNA, the RIG-I double CARD domain forms a PPI with the MAVS N-terminal CARD domain, which is present as only a single CARD motif. Despite both RIG-I and MAVS possessing CARD domains, sequence comparison via BLASTp reveals no significant sequence similarity between the RIG-I and MAVS CARDs. Nonetheless, we used the BiFC assay to determine if arenavirus Z proteins interact with human MAVS N-terminal residues 1-150, containing the CARD domain and the first fifty residues of the proline-rich region (PRR) (Figure 11). Interestingly, all four arenavirus Z proteins under study also interact with MAVS CARD + PRR fragment. Moreover, Guanarito virus Z protein produced a modest signal and Junin virus Z protein produced the largest signal, similar to the results observed with RIG-I CARD. Arenavirus Z proteins have been recently reported to be capable of binding to MDA5

CARD domain, and so our results are further suggestive that a general CARD-binding capability may be a property of at least several arenavirus Z proteins. Once we prepare BiFC constructs for the Z proteins from the non-human pathogens Mobala and Bear Canyon arenaviruses, we will test for their interaction with human MAVS(1-150) to determine if the presence of a Z protein – MAVS interaction is correlated to known pathogenicity, as it appears to be for the interaction with RIG-I and perhaps MDA5.

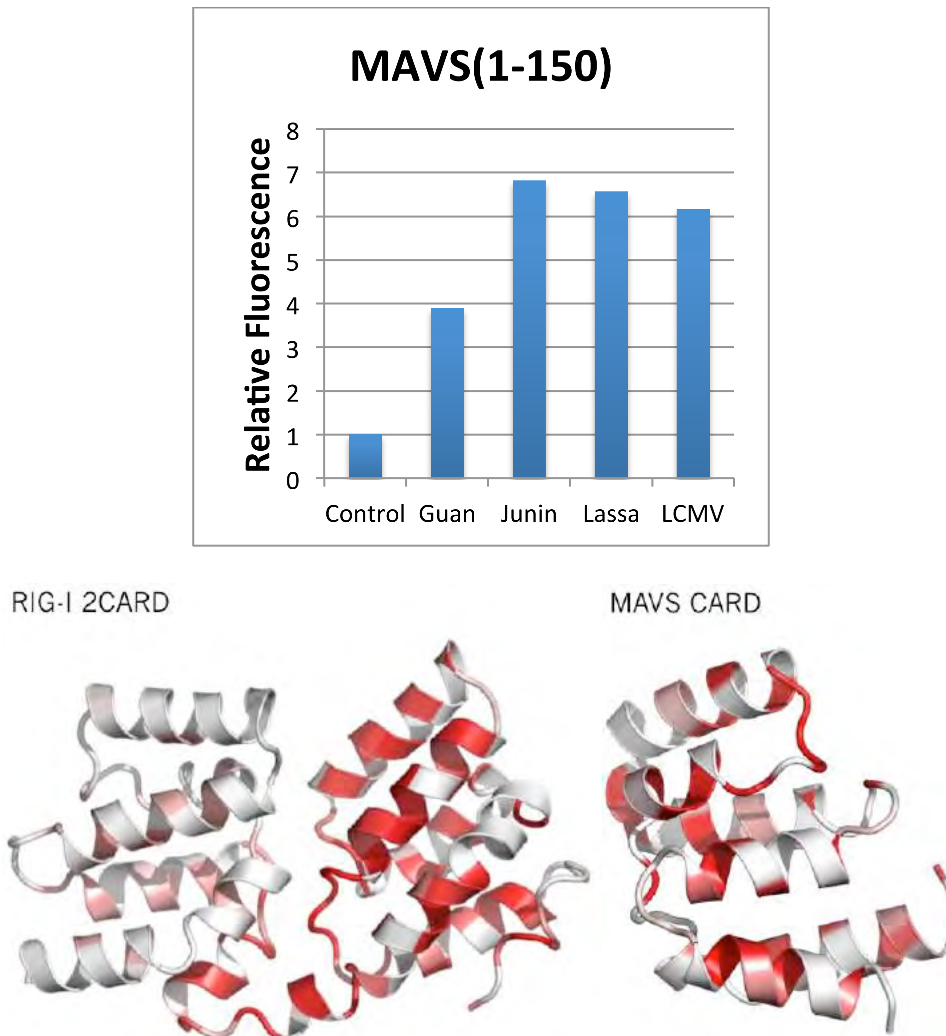

Figure 11. BiFC assay showing interactions of arenavirus Z-proteins with human MAVS(1-150) as detected by normalized fluorescence. Fluorescence activity occurs upon interaction of Z protein with MAVS(1-150). Left: Normalized BiFC fluorescence for MAVS(1-150) and Guanarito (abbreviated Guan) and Junin are NW arenaviruses and Lassa and LCMV are OW arenaviruses. The control is a measure of fluorescence in the absence of PPIs. Right: The MAVS CARD domain structure is displayed color-coded according to sequence conservation (red: high; white: low).

Arenavirus Z protein conclusions

- Both OW and NW arenavirus Z proteins bind hRIG-I.
- Both OW and NW arenavirus Z proteins bind 2CARD domain.
    - In contrast to a previous study (Fan et al, J. Virol., 2010) where OW arenavirus Z proteins did not bind RIG-I.
- OW arenavirus Z proteins may also bind helicase domain (M2H data).
- Guanarito Z protein may have weaker interactions with RIG-I.
    - Sequence analysis identified several Z protein residues important for differentiation of binding.
- Arenavirus Z proteins also interact with other CARD domains present in other host proteins (i.e., MDA5, MAVS) in the same interferon response pathway.

MDA5 – Paramyxovirus V Protein Interaction Bioinformatics and Predictions

MDA5 is a RIG-I-like receptor. It plays a similar role as RIG-I in detecting the presence of a RNA virus infection and then propagating a signal resulting in IFNB induction as part of an initial innate immunity response. MDA5 has a preference for binding blunt-ended dsRNA. A crystal structure of the minimal PPI formed between the V protein of the paramyxovirus parainfluenza virus 5 (PIV5, or simian virus 5, SV5) and pig MDA5 helicase domain was recently determined (Motz et al. 2013, Science, 339:690). Unexpectedly, the PPI interface between PIV5 V protein and MDA5 results from a partial unfolding of the MDA5 helicase domain, separating a β-sheet region into two halves, and thus exposing a region of MDA5 that is normally buried within the MDA5 protein (Figure 12). The PIV5 V protein exploits this newly exposed and normally buried MDA5 region by substituting a two β-strand hairpin from the V protein to replace the MDA5 β-strands displaced from this β-sheet. The end result is a disruption of MDA5's ATP-hydrolysis site, thereby preventing RNA-bound MDA5 from forming its active signaling state as a filament.

Figure 12. Ribbons schematic of the MDA5 binding domain of PIV5 V protein (*left*), with the residues that interact with MDA5 colored cyan. Ribbons schematic of the minimal protein-protein interaction (PPI) of pig MDA5 helicase domain (green) and PIV5 V protein residues 168-219 (cyan) (PDB: 2I1S; *right*). At the core of the PPI is the β-sheet composed of three strands from MDA5 and two strands from a β-strand hairpin motif in PIV5 V protein. The two zinc atoms (grey spheres) plus coordinating residues within V protein are also displayed. Note that the V protein has to unfold to form the complex with MDA5.

Knowledge of the MDA5 – V protein interface allows the respective amino acid sequences of MDA5 and V protein orthologs to be analyzed to predict the likelihood that a specific MDA5 and V protein may form a PPI, and therefore inhibit MDA5-mediated INFB induction. Importantly, lacking the 3-D structure of the MDA5 – V protein complex, a productive sequence analysis would have been extremely difficult or impossible, due to the substantial conformational changes that occur upon formation of the complex. An example of structure and sequence based predictions of MDA5 – V protein interactions follows. Several paramyxovirus V protein sequences were aligned (Figure 13), using representative viruses with different levels of human pathogenicity, from very low to very high, and with different natural host reservoirs. Based upon the MDA5 – PIV5 V protein complex crystal structure, the C-termini of the V protein orthologs were focused upon for this analysis. Specifically, PIV5 V protein residues participating in significant contacts with pig MDA5 in the crystal structure were first identified. Then, these key interaction residues were mapped onto a V protein multiple sequence alignment in order to determine their conservation levels. Of interest were those V protein residue positions that displayed differences in amino acid type or size, and thus may impact the ability of a V protein ortholog to interact with a specific MDA5 ortholog. Ultimately, these residues of interest were mapped back onto the representative MDA5 – V protein 3-D structure (Figure 14). The combination of sequence and 3-D structure greatly enhances the ability to generate host-tropism predictions. For example, W179 in PIV5 V protein is inserted into a cleft in pig MDA5 in

context of the PPI. Sendai virus V protein contains a glutamate residue at this position. This change in both residue size and chemical type (i.e., hydrophobic to negatively charged) would be expected to result in significantly different interactions between these two respective V proteins and pig MDA5. Sequence analysis of the relevant region of MDA5 proteins from known and putative hosts demonstrates that the MDA5 residues predicted to interact with V proteins are highly conserved (Figure 15). The few MDA5 residues in this region that display variability are either conserved substitutions or where the residue side chain doesn't form a direct interaction with V protein, based upon the PIV5 V protein – pig MDA5 crystal structure.

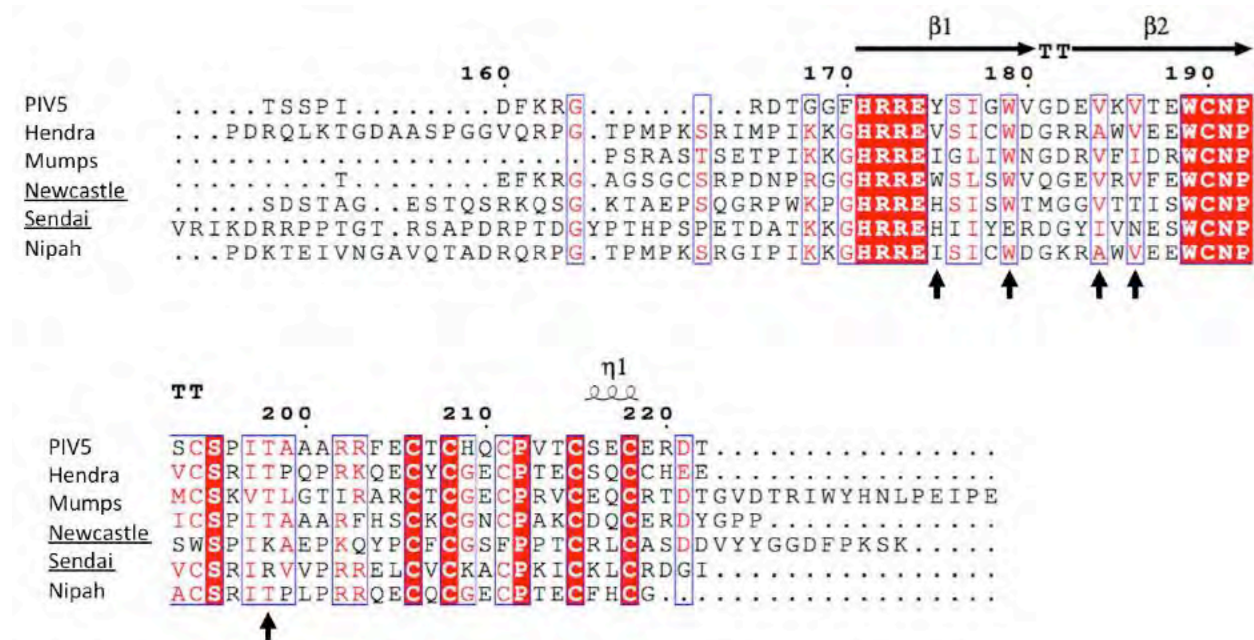

Figure 13. Alignment of C-terminal regions of several representative paramyxovirus V proteins. The indicated secondary structure is based upon the MDA5 – PIV5 V protein complex crystal structure. The black arrows indicate residue positions that are both present at the MDA5 – PIV5 V protein PPI interface and display variation in type or size of the observed amino acids across V proteins, suggesting that they may be determinates for binding to specific MDA5 orthologs.

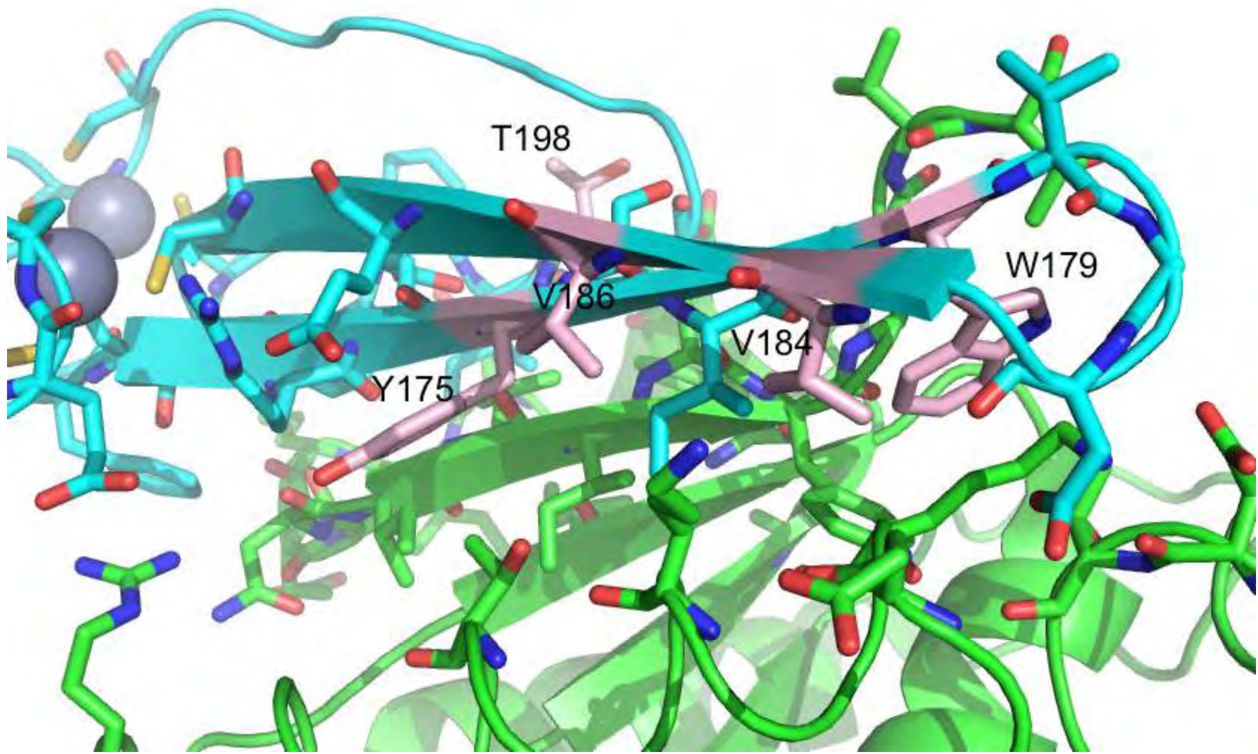

Figure 14. The pig MDA5 (green) – PIV5 V protein (cyan) interface with residues directly participating at the interface displayed as sticks. V protein residue positions predicted to be determinates of V protein recognition and binding to specific MDA5 orthologs are indicated in rose. These residues are variable across V proteins, and change of residue size or type will likely alter or interfere with complex formation.

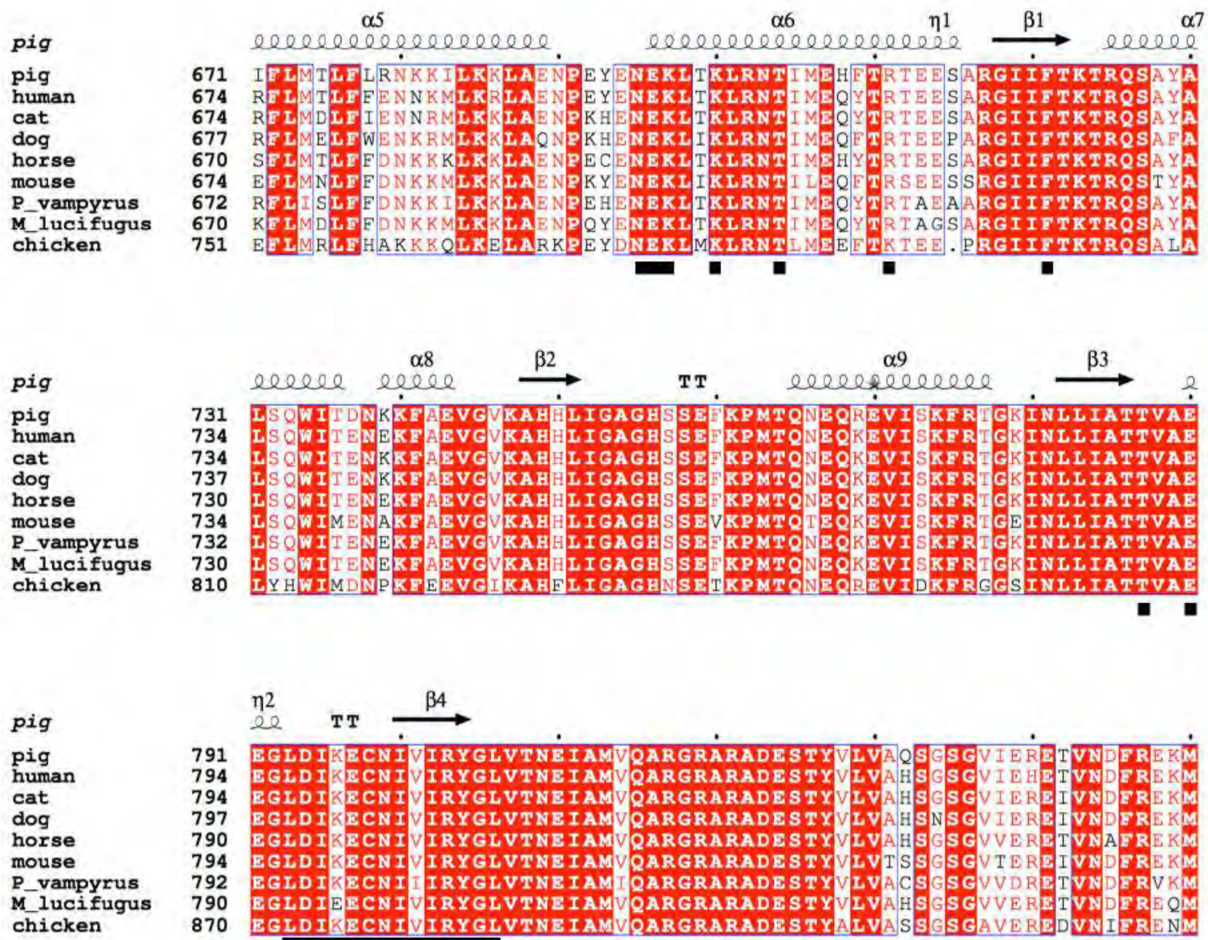

Figure 15. Sequence comparison of host and putative host MDA5 sequences. MDA5 residues (black bars) predicted to directly interact with V proteins, based on PDB 4I1S (pig MDA5 and PIV5 V protein complex). Only three positions predicted to participate in the PPI display any variation, and they are likely not important in dictating MDA5-V protein interactions. They are either conserved substitutions or for K796 (analogous E795 in *M. lucifugus*) the residue's side chain doesn't interact with V protein in the crystal structure.

Paramyxovirus V protein conclusions

- Formation of the V protein – MDA5 complex requires partial unfolding of the respective interaction domains. This likely places constraints upon genetic mutation so as to preserve the respective native fold and function of each protein.
- MDA5 residues at the putative interface are highly conserved.
  - These residues are integral for *normal* MDA5 structure and activity.
  - Thus, MDA5 likely does not represent a barrier to viral adaptation to a new host (excluding the possibility of different species-specific post-translation modifications).
- Paramyxovirus V protein residues at the putative interface are only partially conserved

- Thus, this V protein sequence variation may alter the formation of MDA5-V protein complexes with a concomitant variation in IFNβ induction.
- The MDA5-V protein complex is a possible barrier to host expansion, but is likely only a low barrier to change in host tropism.

Influenza A PB2 – Human MAVS interaction

A peer-reviewed manuscript was published during this reporting period (Patel, D., Schultz, L.W., and Umland, T.C., Influenza A polymerase subunit PB2 possesses overlapping binding sites for polymerase subunit PB1 and human MAVS proteins. Virus Res., 172, 75-80 (2013)). This publication is summarized here:

The Orthomyxovirus Influenza A virus is an important human pathogen accounting for widespread morbidity and mortality, with new strains emerging from animal reservoirs possessing the potential to cause pandemics. The influenza A RNA-dependent RNA polymerase complex consists of three subunits (PA, PB1, and PB2) and catalyzes viral RNA replication and transcription activities in the nuclei of infected host cells. Sequence variations in the PB2 subunit has been implicated in pathogenicity and host adaptation. This includes the inhibition of type I interferon induction through interaction with the host's mitochondrial antiviral signaling protein (MAVS), an adaptor molecule of RIG-I-like helicases. This study reported the identification of the cognate PB2 and MAVS interaction domains necessary for complex formation. This result was achieved by co-expression of PB2 and MAVS full-length and fragment constructs in mammalian cells, and detecting the presence of PB2-MAVS complex by co-immunoprecipitation and western blot analysis. Specifically, human MAVS residues 1-150, containing both the CARD domain and the N-terminal portion of the proline rich-region, and influenza A/WSN/1933 (H1N1) PB2 residues 1-37 are essential for PB2-MAVS virus-host protein-protein complex formation (Figure 16). The three α-helices constituting PB2(1-37) were tested to determine their relative influence in complex formation, and Helix 3 was observed to promote the primary interaction with MAVS. PB2's MAVS-binding domain unexpectedly coincided with its PB1-binding domain, indicating an important dual functionality for this region of PB2. Analysis of these interaction domains suggests both virus and host properties that may contribute to host tropism. Additionally, the results of this study suggest a new strategy to develop influenza A therapeutics by simultaneously blocking PB2-MAVS and PB2-PB1 protein-protein interactions and their resulting activities.

Influenza A PB2 conclusions

- The influenza A PB2 – MAVS minimal binding interaction involves a domain from each protein (PB2 N-terminal domain and MAVS CARD domain) that participates in other functionally important PPIs.
    - The PB2 N-terminal domain participates in a critical interaction with Influenza A PB1 as part of the formation of the viral polymerase complex in the host cell nucleus.

- The MAVS CARD domain interacts with the RIG-I 2CARD domain as part of an antiviral interferon response signaling pathway.

- Influenza A PB2 inhibits the RIG-I interferon response pathway by preventing the formation of the MAVS – RIG-I complex in the cytoplasm.
- Cellular location (cytoplasm versus nucleus) dictates, in part, influenza A PB2's function.
- These results suggest a new strategy to develop influenza A therapeutics by simultaneously blocking PB2-MAVS and PB2-PB1 protein-protein interactions and their resulting activities.

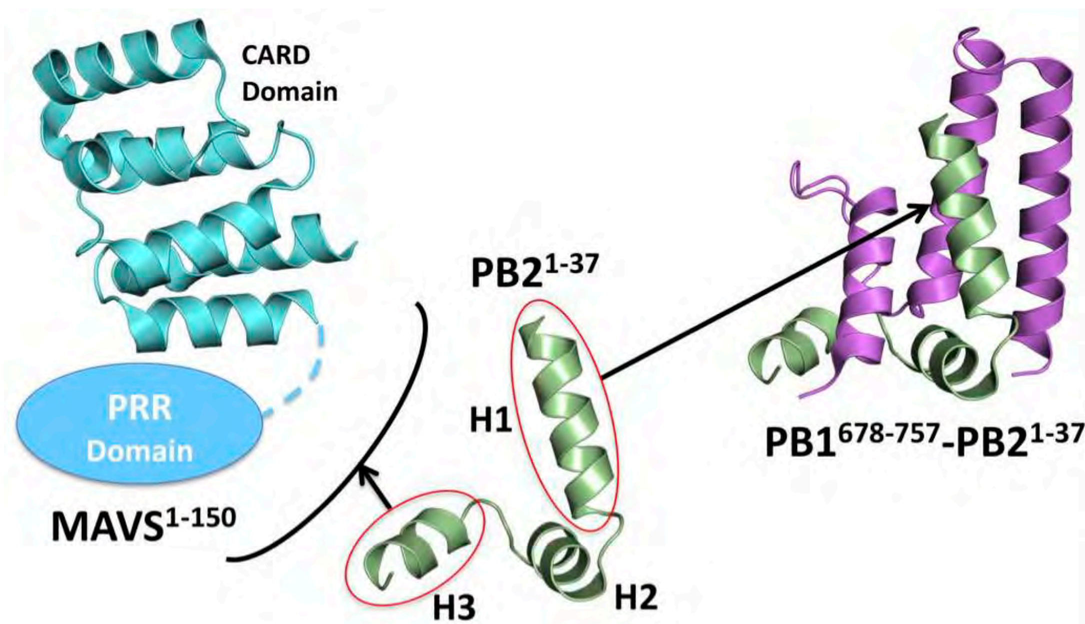

Figure 16. Minimal interaction domains of influenza A/WSN/1933 (H1N1) PB2 and human MAVS has been determined in the Umland Lab, revealing that the same PB2 domain also mediates PB1:PB2 complex formation. Mapping of the presence of the PB2:MAVS interaction across strains and potential hosts is underway.

RIG-I oligomeric state analysis and arenavirus Z protein interaction

The screening for interactions between arenavirus Z proteins and human RIG-I demonstrated that while Z proteins interacted with full-length RIG-I, the interaction was enhanced with the isolated RIG-I double CARD domain. In the absence of viral RNA, RIG-I is in an inactive conformational state, where it is thought to protect its double CARD domain from participating in PPIs – primarily the productive interaction with MAVS – but presumably also from Z proteins in the absence of a viral RNA. To further explore this, we have analyzed human RIG-I constructs (double CARD domain, residues1-228; helicase-RBD, residues 241-925; and the full-length RIG-I, residues 1-925) by small angle X-ray scattering (SAXS) and analytical size exclusion chromatography (SEC).

SAXS data was collected at Beamline 4-2 at the Stanford Synchrotron Light Source. We have been able to construct models of the human RIG-I full length and helicase-RBD domains using our SAXS data combined with the crystal structure of the duck RIG-I structure recently published (Kowalinski, Lunardi et al. 2011). The duck structure is a dimer in the crystal which fits well into our molecular envelope reconstruction of the full length human RIG-I in solution by SAXS (Figure 17). SEC data indicate that full-length RIG-I runs as a monomer in the presence of RNA but as a dimer in its absence (data not shown), suggesting that viral RNA causes the oligomeric state of RIG-I to change from dimer to monomer and presumably exposing the double CARD domain for participation in PPIs.

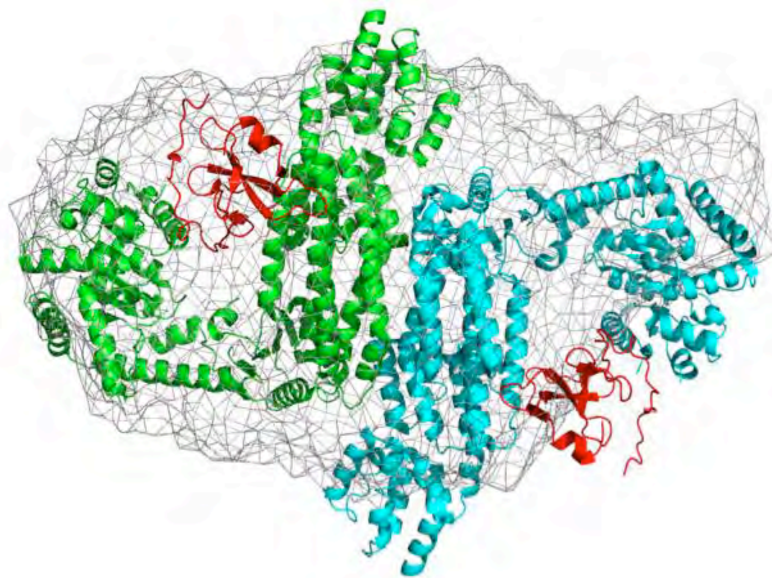

Figure 17. SAXS molecular envelop of unligated full-length RIG-I with the crystal structure of the dimeric CARD-Helicase domains of RIG-I plus the RBD (red) fit into the surface reconstruction.

What opportunities for training and professional development has the project provided?
If the research is not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report." Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Multiple training and professional development opportunities currently exist in conjunction with this project. Dr. Schultz supervised Jennifer Breen (Research Associate) and Kristin Sutton, Ph.D. (Postdoctoral Associate). (Dr. Schultz departed HWI in February 2015 to become Lead Project Manager at QuadPharma, with oversight on cGMP biologics manufacturing. Dr. Umland assumed supervision of Breen and Sutton at that time.) They are receiving training and experience in small-angle x-ray scattering, mammalian cell culture, mammalian two-hybrid technology, BiFC, and protein expression and purification. Both regularly attend and participate in HWI's weekly institute-wide research meeting, and research seminars presented by prominent invited speakers are regularly available at both HWI and at other organizations on the Buffalo-Niagara Medical Campus (e.g., University at Buffalo, Roswell Park Cancer Institute).

The Schultz Lab hosted an undergraduate intern over this reporting period. Ryan O'Conner (Junior, SUNY, University at Buffalo) began his undergraduate internship during the fall 2013 academic semester, and continued through the 2013-2014 academic year and into summer 2014. He received training in virology, molecular biology, and protein chemistry, and he presented his research studies at the HWI-UB-RPCI Summer Undergraduate Biomedical Research Day (August 1, 2014).

Dr. Umland supervises Jessica Graham (Research Associate), and as noted above assume supervision of Breen and Sutton upon Schultz's departure from HWI. Graham is receiving training in biochemical activity assays and cell culture. Graham regularly attends and participates in HWI's weekly institute-wide research meeting, and research seminars presented by prominent invited speakers are available for professional development at both HWI and at other organizations on the Buffalo-Niagara Medical Campus (e.g., University at Buffalo, Roswell Park Cancer Institute).

The PIs (Schultz and Umland) engaged in a variety professional development activities. Drs. Schultz and Umland both were active participants in HWI's weekly institute-wide research meeting, including each presenting their respective lab's current research results. They attended research seminars presented by prominent invited speakers at HWI and at other organizations on the Buffalo-Niagara Medical Campus (e.g., University at Buffalo, Roswell Park Cancer Institute).

Schultz attended 2014 American Crystallographic Association Meetings in Albuquerque, NM,. This is the premier research meeting in North America for biological X-ray crystallography. Several sessions provided continuing education in small-angle X-ray scattering relevant to this project.

Umland participates in the University at Buffalo's weekly Infectious Disease Research Conference, a university-wide group of researchers and clinicians specializing in infectious diseases. He presented on-going research to this group in March 2015.

Umland, Sutton, Graham, and Breen all participated in the BioXFEL Crystallization Workshop and the public scientific seminar sessions of the following BioXFEL NSF Science and Technology Center (STC) evaluation site visit (June 2-4, 2015). The BioXFEL STC is an NSF funded multi-site center, with HWI as a major participant, with the goal of developing and disseminating optimal methodologies for employing new free-electron laser technology that produces intense X-ray pulses towards transformational advances in structural biology.

How have the results been disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."
Describe how the results have been disseminated to communities of interest. Include any outreach activities that have been undertaken to reach members of communities who are not usually aware of these research activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Results have been disseminated to communities of interest through several mechanisms.

Publication:

Patel, D., Schultz, L.W., and Umland, T.C., Influenza A polymerase subunit PB2 possesses overlapping binding sites for polymerase subunit PB1 and human MAVS proteins. Virus Res., 172, 75-80 (2013).

Scientific Presentations:

L. Wayne Schultz, Joseph R. Luft, Eleanor Cook, George T. DeTitta, Angela M. Lauricella, Raymond M. Nagel, Edward H. Snell, Jennifer R. Wolfley, *High-throughput crystallization of cytosolic and membrane bound proteins*. Eighth Annual Fragment-Based Drug Discovery: From Discovery to Lessons Learned. April 16-17, 2013, San Diego, CA.

Timothy C. Umland, Joseph R. Luft, Eleanor Cook, George T. DeTitta, Angela M. Lauricella, Raymond M. Nagel, Edward H. Snell, Jennifer R. Wolfley, *High-throughput crystallization of cytosolic and membrane proteins to structurally enable drug discovery*. 7th Drug Design & Medicinal Chemistry Conference. May 8-10, 2013, Boston, MA.

Umland, T.C., Patel, D., and Schultz, L.W., *Influenza A PB2 and human MAVS complex: extending crystal structural data to identify virus-host protein-protein interaction determinates of host tropism*. Pittsburgh Diffraction Conference, Buffalo, NY, September 18-20, 2013.

Umland, T.C. and Schultz, L.W., *Cross-species virus-host protein-protein interactions inhibiting innate immunity*. 2013 DTRA Basic Research Technical Review, Springfield, VA, July 30, 2013.

Umland, T.C. and Schultz, L.W., *Medical Countermeasures*. Weill-Cornell Medical College, New York, NY, May 6, 2014.

Umland, T.C. and Schultz, L.W., *Cross-species virus-host protein-protein interactions inhibiting innate immunity*. 2014 DTRA Basic Research Technical Review, Springfield, VA, July 21, 2014.

O'Conner, R., Umland, T. and Schultz, L.W., *Arenavirus Z protein interactions with RIG-I to inhibit innate immune responses*. HWI-UB-RPCI Summer Undergraduate Biomedical Research Day, Buffalo, NY, August 1, 2014.

Umland, T.C., *New approaches to infectious diseases*. University at Buffalo Infectious Disease Conference. Buffalo, NY March 12, 2015.

Umland, T.C. and Schultz, L.W., Cross-species virus-host protein-protein interactions inhibiting innate immunity. 2015 Defense Threat Reduction Agency (DTRA) Basic Research Technical Review, Springfield, VA, July 20, 2015.

Outreach activities:

This project was prominently highlighted on HWI's home page (Summer 2013). This web-presence provides an avenue for outreach to both the scientific community and the lay community interested in biomedical science. It also is an indication of HWI's institutional support for this project.

Accompanying this announcement on the HWI home page is a link to an article describing our project (www.hwi.buffalo.edu/news_events/February/2_2013.html). This project was the cover story for HWI's Winter 2013 newsletter (www.hwi.buffalo.edu/newsroom/Newsletter/Winter_2013.pdf). Hardcopies of this report are distributed to a mailing (1700) and email (700) list that includes both members of the scientific community and the general

An article entitled Hauptman-Woodward Researchers Granted $1.1M for Virus Study appeared in the February 12, 2013 issue of Business First, a print (circulation 9000) and online (subscriptions 6300) business journal. The article describes the main goals of the project and why it is important to public health. A link to the article follows:
http://www.bizjournals.com/buffalo/news/2013/02/12/researchers-granted-11m-for-virus.html

Dr. Umland was invited to participate in the founding of the SUNY REACH (State University of New York - Research Excellence in Academic Health) Infectious Diseases Focus Group. SUNY REACH is a program to unify and advance the research vision of New York State Academic Health Centers and their integrated Medical Schools. Dr. Umland participated in the initial organizational meeting of the Infectious Diseases Focus Group at Stony Brook University, June 19-20, 2013. Representatives from the flagship SUNY universities (University at Buffalo, Stony Brook, SUNY Downstate Medical Center, and SUNY Upstate Medical Center) were also present at this meeting. Dr. Umland presented an overview of the DTRA supported project described in this report, with the goal of both raising awareness of the research project across SUNY and to identifying potential collaboration partners for future related projects.

Schultz and Umland visited Weill-Cornell Medical College (WCMC; May, 2014), and met individually with 8 faculty members, plus in a group meeting of > 30 WCMC researchers to explore areas of possible collaboration. This visit was conducted in conjunction with Engility Corporation as part of a consortium recently awarded a Combating Weapons of Mass Destruction (CWMD) Research and Technology Development Indefinite Delivery/Indefinite Quantity (IDIQ) contract (HDTRA1-14-D-0004).

As part of the Engility consortium, Dr. Schultz and Dr. Umland responded with capability statements to DTRA RFIs on Medical Countermeasures: Development of Therapeutics and Vaccines and Epitope Mapping.

Umland met with Insyte Consulting leadership and staff in 2013 to discuss research capabilities and current projects. Insyte Consulting's mission includes assisting Western New York technology and manufacturing companies to transition cutting edge technical and scientific discoveries into products and services.

Schultz and Umland were featured on a local TV news broadcast (May 2014; WIVB) on the public health implications of zoonotic viruses acquiring new host species, including humans. This news story was prompted by the emergence of the Middle Eastern Respiratory Syndrome (MERS) virus as a human pathogen.

Schultz and Umland were featured in the HWI Spring 2014 Newsletter, which is distributed to >2400 individuals and companies. This audience is primarily lay people who are interested in biomedical research and structural biology. The article's focused upon Schultz and Umland's participation in a consortium lead by Engility Corporation the recently was awarded (December 2013) an Indefinite Duration / Indefinite Quantity contract from DTRA for Combating Weapons of Mass Destruction.

Dr. Schultz presented a workshop on crystallization of proteins to Willow Ridge elementary school children and Boy Scouts in April 2014 as part of a effort to inspire interest in STEM programs and learning.

Umland and Schultz met with local and regional biotech business personnel to discuss research capabilities and current projects. A result of these discussions is a joint project with a biotech startup (Terapore Technologies) to improve protein purification technology, especially for difficult to isolate proteins, using engineered membranes rather than standard chromatography bulk media. Many viral and eukaryotic proteins fall into the difficult to isolate category, playing heavily into formation of this collaboration.

Schultz and Umland made presentations to local groups with general interests in biomedical research as part of HWI's and the Buffalo Niagara Medical Campus outreach to the general public.

What do you plan to do during the next reporting period to accomplish the goals?
If there are no changes to the agency-approved application or plan for this effort, state "No Change." Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

No Change.

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